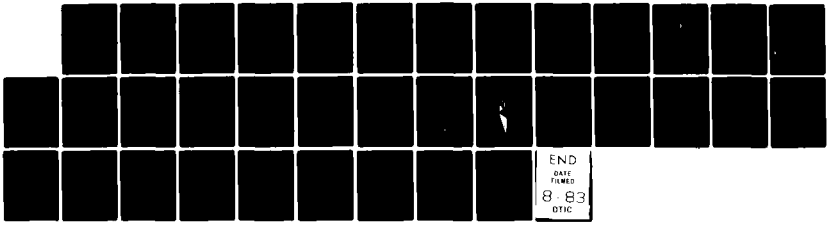
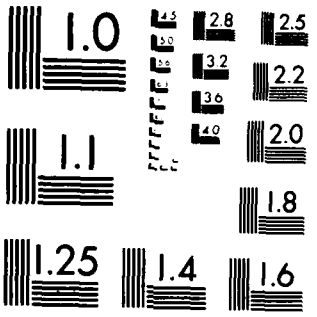
AD-A130 161 A PORTABLE MICROPROCESSOR CONTROLLED MULTICHANNEL FLUOROMETER FOR MARINE..(U) EMORY UNIV ATLANTA GA DEPT OF CHEMISTRY P OLDHAM ET AL. 13 JAN 83 1/1

UNCLASSIFIED EMORY/DC/TR-83/1 N00014-83-K-0026 F/G 14/2 NL





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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: EMORY/DC/TR-83/1	2. GOVT ACCESSION NO.: AD-A130161
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): A Portable, Microprocessor Controlled, Multichannel Fluorometer for Marine Analysis	5. TYPE OF REPORT & PERIOD COVERED: Interim Technical Report
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Philip Oldham, Gabor Patonay and Isiah M. Warner	8. CONTRACT OR GRANT NUMBER(s): N00014-83-K-0026
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Chemistry, Emory University, Atlanta, Georgia 30322	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: NR-051-841
11. CONTROLLING OFFICE NAME AND ADDRESS: Chemistry Program, Office of Naval Research, 800 Quincy St. - Arlington, VA 22217	12. REPORT DATE: June 13,1983 13. NUMBER OF PAGES: 33
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office): Glenn Daniel Oldre, 206 O'Keefe Building, Atlanta, Georgia 30332	15. SECURITY CLASS. (of this report): Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for Public Release: Distribution Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Prepared for Publication in Deep Sea Research

A

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Fluorescence, Marine Analysis, Portable Fluorometer, Rapid Scanning Fluorometer, Chlorophyll Analysis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The design of a portable, multichannel fluorometer which provides enhanced sensitivity and rapid data acquisition is described. The advantages of multidimensional fluorescence detection are discussed with special reference to the continuous monitoring of in vivo chlorophyll fluorescence in the marine environment. Sensitivity, detection limit, linearity of detection, and other parameters are used to evaluate the instrument design. Preliminary experiments are presented in regard to chlorophyll determinations in the open ocean.

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OFFICE OF NAVAL RESEARCH

Contract N00014-83-K-0026

Task No. NR 051-747

TECHNICAL REPORT NO. 1

A Portable, Microprocessor Controlled, Multichannel Fluorometer for Marine Analysis

by

Philip Oldham, Gabor Patonay

and Isiah M. Warner

Prepared for Publication

in

Deep Sea Research

Department of Chemistry
Emory University
Atlanta, Georgia 30322

June 1983

ABSTRACT

The design of a portable, multichannel fluorometer which provides enhanced sensitivity and rapid data acquisition is described. The advantages of multidimensional fluorescence detection are discussed with special reference to the continuous monitoring of *in vivo* chlorophyll fluorescence in the marine environment. Sensitivity, detection limit, linearity of detection, and other parameters are used to evaluate the instrument design. Preliminary experiments are presented in regard to chlorophyll determinations in the open ocean.

INTRODUCTION

Fluorescence spectroscopic techniques are characterized by exceptional sensitivity and selectivity. The sensitivity of fluorescence is typically three to four orders of magnitude better than that of absorbance. This advantage arises from the direct detection of photons in fluorescence in contrast to the measurement of a small difference in two large photon signals in absorbance.

The multiparametric nature of fluorescence spectroscopy provides the advantage of excellent selectivity. Emission intensity is dependent on both the wavelengths of excitation and emission. Many fluorophores can be spectrally separated based on these two parameters alone. However, by incorporating other parameters such as fluorescence lifetimes, phosphorescence spectra, and polarization, even greater selectivity can be obtained.

Many recent studies have cited the multiparameter advantage of luminescence spectroscopy and have utilized it in the analysis of multicomponent samples. For example, Ho and Warner (1982) were able to qualitatively differentiate a ternary mixture of polynuclear aromatic hydrocarbons by their respective excitation spectra, emission spectra, and phosphorescence lifetimes. The spectral deconvolution of a multicomponent sample by selective quenching was reported by Fogarty and Warner (1982). Vo-Dinh et al. (1981) and others (Eastwood, 1981; Lloyd, 1980) have demonstrated the utility of synchronous scanning fluorescence with a priori knowledge of multicomponent sample composition. Inman and Winefordner (1982) have enhanced the

selectivity of synchronous scanning by maintaining a constant energy difference ,$\Delta\nu$, between excitation and emission wavelengths. Several other examples can be cited to further emphasize the multiparametric advantage (Ho and Warner, 1982; Vo-Dinh and Martinez, 1981; Hershberger et al., 1981).

Advances in instrument design have been crucial to the development of methods which capitalize on the sensitivity and selectivity of fluorescence. The trend in most areas of instrumentation development is from simple to complex and fluorescence instrumentation has not been an exception. The early instruments used simple bandpass filters to restrict the wavelengths of excitation and emission. Later, more sophisticated spectrofluorometers incorporated two scanning monochromators for excitation and emission resolution. Both of these instrument types are still well represented in current literature. However, the desire for more data in less time has continued to inspire further developments. Holland et al. (1973) described an instrument capable of simultaneous absorbance and fluorescence measurements. This instrument could correct both excitation and emission spectra for instrumental effects as well as calculate quantum efficiencies using the system computer.

The most recent advances in instrumentation have been facilitated by improvements in computer technology and optoelectronic detection devices. Improved computer technology allows the acquisition and subsequent analysis of large data sets while reducing operator time. The development of imaging devices such as vidicons, charge-coupled devices, and linear photodiode arrays has provided simultaneous

multiwavelength detection. Warner et al. (1979) and Johnson et al. (1979) combined these two technologies in the video fluorometer (VF) which provided a new level of sophistication and versatility in spectrofluorometers. Due to its unique two-dimensional detection scheme, the VF can acquire a "total luminescence" spectrum in a few seconds or less (Wong et al., 1982).

The linear photodiode arrays and intensified arrays are the newest of the multichannel detectors and offer several advantages over the older vidicons. These arrays are usually constructed as one-dimensional detectors in contrast to the two-dimensional vidicons. However, their good sensitivity, minimal lag, and minimal blooming provide superior performance for many applications (Dessey and Nunn, 1976; Ingle and Ryan, 1981). Two-dimensional photodiode arrays are also available but current technology has not advanced sufficiently to warrant routine use in spectroscopic applications.

Advances in instrumentation along with the advantages of sensitivity and selectivity have established fluorescence spectroscopy as an important tool in the determination of many inorganic and organic species. This is particularly true in the study of biological systems where highly conjugated fluorescent molecules are commonly found. Lorenzen (1966) demonstrated that fluorescence is a sensitive method for the measurement of in vivo chlorophyll a in the open ocean. He was able to detect as little as 10^{-11}M chlorophyll by using a simple Turner model III fluorometer equipped with a red sensitive photomultiplier tube (PMT). This approach to chlorophyll monitoring has become increasingly popular (Heaney, 1978; Slovacek and Hannan,

1977; Kiefer, 1973) because of capabilities for continuous data acquisition. The previous methods involved discrete sampling, filtering, extraction, and subsequent analysis by either the trichromatic method of light absorbance (Richards and Thompson, 1952) or fluorescence (Yentsch and Menzel, 1963). These techniques were much more time consuming and offered much less topographical information.

A few recent studies have attempted to use fluorescence selectivity to identify specific pigments characteristic of certain algal groups (Moreth and Yentsch, 1970; Marker and Jinks, 1982). Yentsch and Yentsch (1979) were able to make gross characterization of phytoplankton populations by spectral differences in excitation spectra. This indicates that a significant amount of spectral information is undetected in continuous in vivo studies by conventional instrumentation. Therefore a new fluorometer is required to provide rapid data acquisition, sensitivity, and portability along with multiwavelength detection.

This paper presents the design of a portable, multichannel fluorometer (PMF) for use on board ship for analysis of fluorescent species in the ocean. Preliminary laboratory evaluation and experimental results will be discussed with regard to applications in the marine environment. The potential for shipboard operation in the continuous monitoring of in vivo chlorophyll a excitation and emission spectra will be described along with the apparent detection limits and an explanation of the necessary instrumental parameters.

MATERIALS AND METHODS

Samples

Standard chlorophyll a was obtained from Sigma Chemical Company and the Chlamydomonas reinhardi from Carolina Biological Supply. The real, extracted chlorophyll a, samples were collected on the November, 1982 cruise of the R.V. Gyre in the Gulf of Mexico*. Sample collection required filtering approximately 500ml of seawater through a Whatman GF/C filter. The filters were frozen for transport and storage. A tissue grinder facilitated extraction of chlorophyll a into 90% acetone, as described by Yentsch et al. (1963).

Instrumentation

There were six characteristics which necessarily had to be incorporated into the design of the PMF: 1) Portability 2) Sensitivity 3) Rapid data acquisition 4) Multidimensional detection 5) Automation capabilities 6) Rugged construction. Portability is required because many fluorescent species, especially in biological systems, must be investigated in their natural environment. Therefore, the instrument must be easily transportable and capable of operation in a non-laboratory environment. Sensitivity is crucial since concentrations of chlorophyll a in the open ocean are typically 10^{-8}-10^{-11} M. The characteristics of rapid data acquisition and multidimensional detection are needed to provide the spectral information with

*This cruise was in collaboration with Dr. D. R. Schink, Texas A&M University, College Station, Texas.

sufficient time resolution to allow topographical mapping. It is also desirable to incorporate automation capabilities into the design of the instrument since it will often be operating continuously. Finally, the construction of the fluorometer must be sufficiently rugged to withstand transport and rough weather conditions at sea.

RESULTS AND DISCUSSION

Instrument Design

The previously mentioned characteristics of portability, sensitivity, rapid data acquisition, and multidimensional detection were all achieved by using an intensified photodiode array consisting of 512 elements. When coupled to a flat field spectrograph this array can simultaneously detect the spatially dispersed emission spectrum over a 600nm window. The detection system used was Tracor Northern's (Middleton, WI) IDARSS system which consists of an intensified array, spectrograph, and multichannel analyzer.

Multidimensional detection is possible by the rapid acquisition of emission spectra at several different excitation wavelengths (Figure 1). A circular variable filter wheel was purchased from Optical Coating Laboratory (Santa Rosa, CA) to provide the excitation resolution. This filter wheel is an interference filter constructed such that the transmitted wavelengths vary linearly with the angular position of the wheel. The wavelength range is 400-700nm with the transmittance ranging from 20% at 400nm to 46% at 700nm. A 200 step stepping motor was obtained from Superior Electric (Bristol, CT) to drive the filter wheel.

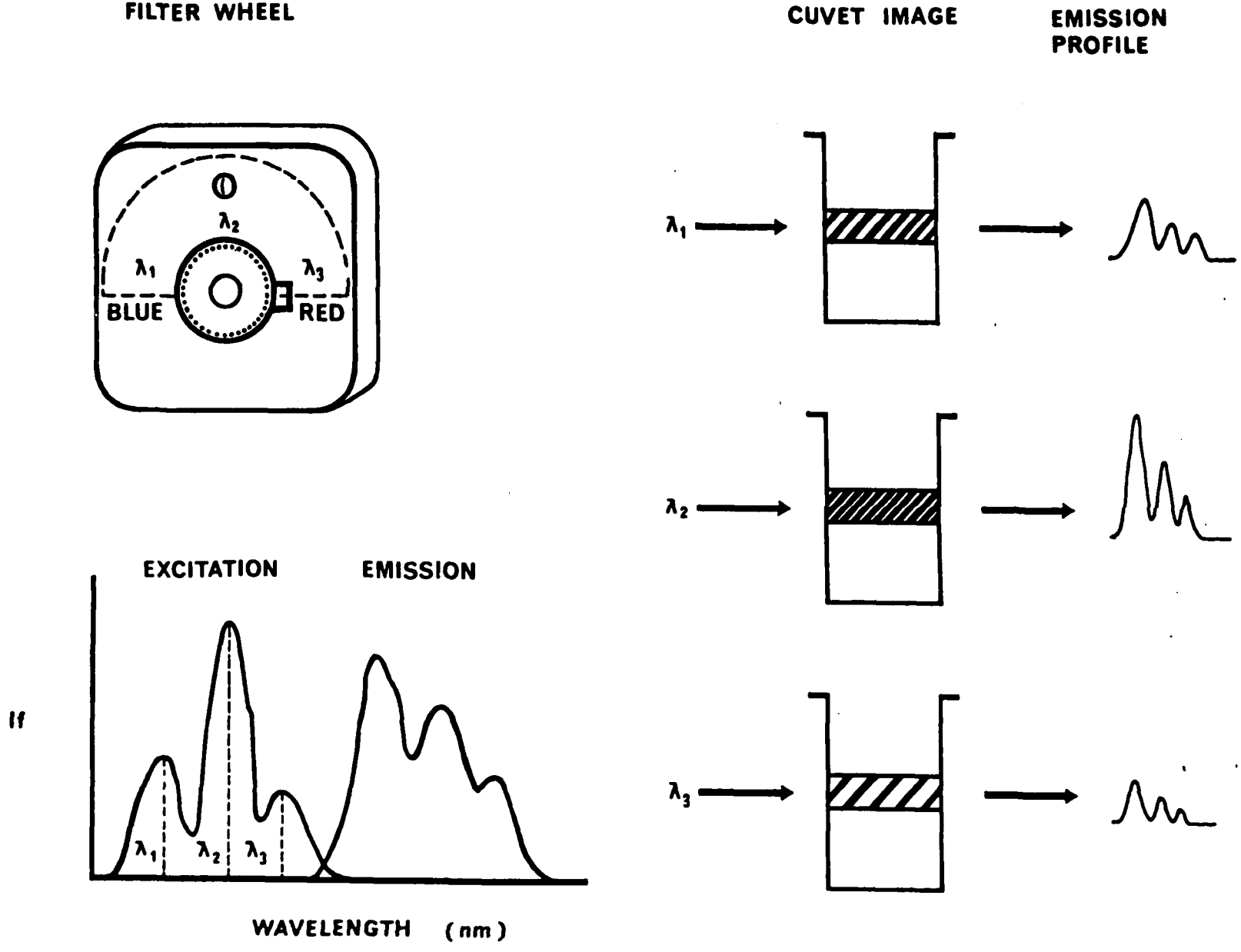
FILTER WHEEL
CUVET IMAGE
EMISSION PROFILE
λ2
λ1
λ3
BLUE
RED
λ1
λ2
λ3
EXCITATION
EMISSION
λ1
λ2
λ3
WAVELENGTH (nm)

FIGURE 1

Both the diode array and the stepping motor driven filter wheel were interfaced to an Apple II+ with 64 Kbytes (8 bits/byte) of RAM, two mini-floppy disk drives, and a CRT monitor. An Apple Super Serial@ interface (Apple Computer, Cupertino, CA) provides the RS-232c serial communication link between the Apple II+ and the TN-1710 multichannel analyzer which controls the diode array. The stepping motor is accessed by a Cybernetic (San Gregorio, CA) CY512 stepping motor controller via a SSM (San Jose, CA) parallel interface. Software control of the stepping motor was provided through the CY512 by ASCII commands from the Apple II+.

Custom built, foam insulated cases with an ABS plastic outer shell protect the intrument in shipment and from environmental conditions during operation. These cases have been modified to accomodate electrical and water connections and to eliminate stray light. A diagram of the completed PMF is given (Figure 2).

Sensitivity

A comparative evaluation of the PMF, VF, and Perkin-Elmer LS-5 was performed. The different modes of detection and signal readout used by the three instruments make a completely equal comparison difficult. However, Talmi (1982) compared the detection sensitivity of a PMT, SIT vidicon, diode array, and intensified array by the calculation of signal-to-noise ratios S/N at peak maxima while approximately equating the total exposure times. Significant improvement of S/N for the arrays resulted from longer integration times and detector cooling with cold water to reduce dark current noise. This method seemed to be the most satisfactory and informative comparison for our purposes and was,

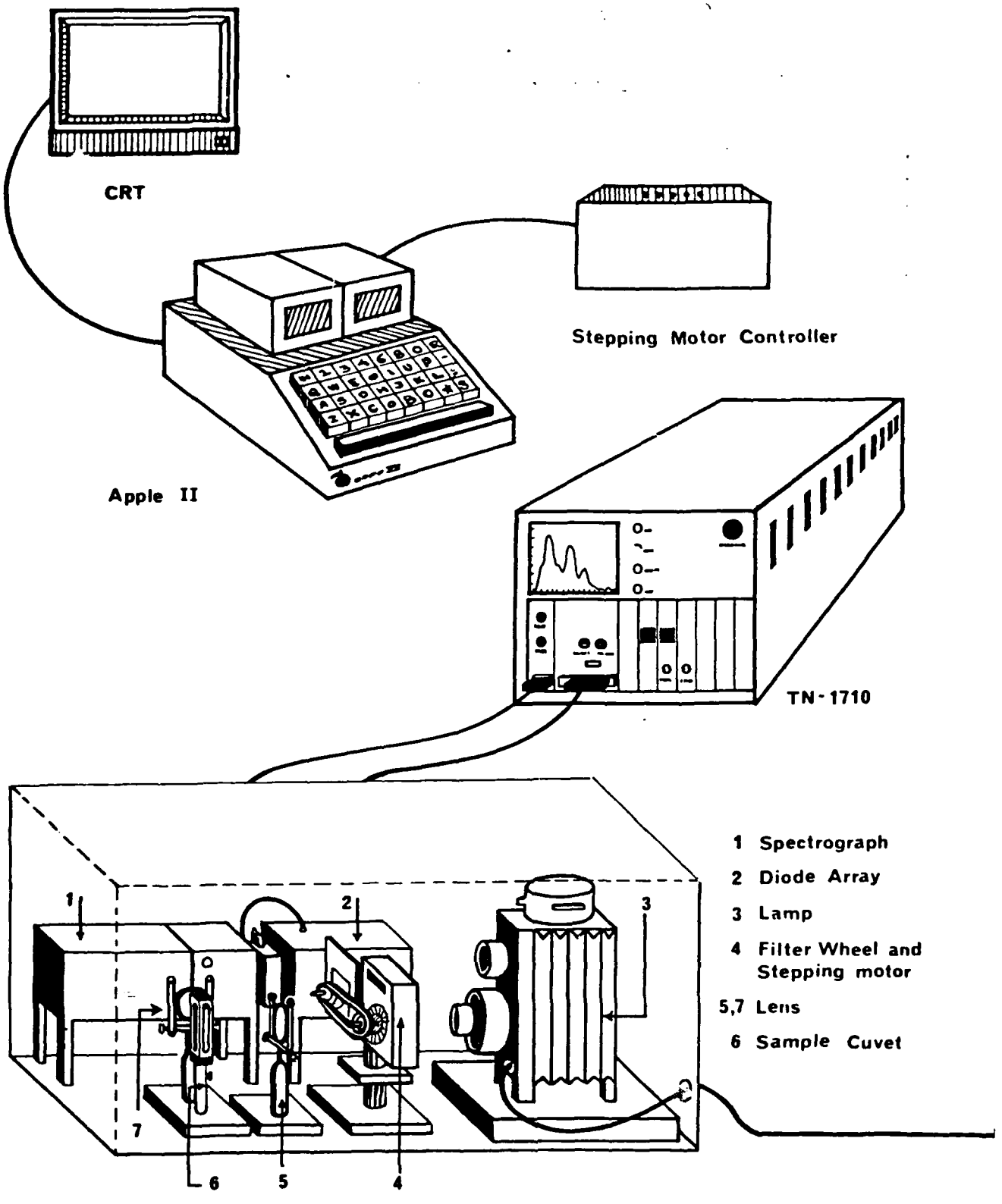
CRT
Stepping Motor Controller
Apple II
TN-1710
1 Spectrograph
2 Diode Array
3 Lamp
4 Filter Wheel and Stepping motor
5,7 Lens
6 Sample Cuvet
1
2
3
4
5
6
7

FIGURE 2

therefore, used in this study.

In order to obtain the S/N values, 100 replicate standard chlorophyll a (10^{-8} M) spectra were acquired. The mean value of the emission maxima was calculated as the signal. The noise at the peak was characterized by the standard deviation of the peak maxima. By calculating the S/N at the peak in this manner both photon flicker and dark current noise contributions were accounted for.

The experimental conditions and the S/N values obtained for the three fluorometers in our laboratory are given in Table I. Exposure times were selected such that a fair comparison could be obtained under relatively normal conditions. As expected from Talmi's (1982) results, the PMF with its intensified array is significantly more sensitive than either the LS-5 or VF. In addition, it must be pointed out that the exposure time for the LS-5 was for a 50nm scan while the PMF covered 600nm and the VF over 200nm in both excitation and emission dimensions. Therefore, both the PMF and VF are relatively more versatile than is apparent from Table 1 due to the multiplex advantage (Busch and Malloy, 1979).

Detection Limit and Linearity

The calibration curve for standard chlorophyll a (Figure 3) shows linear response over three orders of magnitude in concentration with a correlation coefficient of 0.9997. This data was acquired with an exposure time of 0.205 sec./scan. The detection limit given (Figure 3) was calculated for the S/N of 2 by accumulating 30 scans with an exposure of 1 sec./scan. However, this detection limit is somewhat arbitrary due to the dependence of signal on integration time. By

Table 1

Comparison of detection for chlorophyll a

between the portable fluorometer, video fluorometer

and Perkin-Elmer LS-5 *

	Integration Time	Average Signal at Peak (Counts)	Average Noise at Peak (Counts)	S/N At Peak
Portable Fluorometer	30 scans; 0.205 sec/scan ET = 6.15 sec	98,006.28	1120.84	87.44
LS-5	50nm/scan; 8nm/sec ET = 6.25 sec	88.24	1.21	72.93
Video Fluorometer	11 scans; 0.573 sec/scan ET = 6.31 sec	2,113.95	78.52	26.92

* Measurements were performed at peak; λex = 430nm, λem = 667nm

employing longer integration times a proportional reduction in detection limit can be observed. Therefore, the limit of detection falls well below that necessary for open ocean studies.

Computer Control and Data Acquisition

Control of the PMF is achieved using Applesoft BASIC software with some simple task-specific 6502 machine language subroutines. Communication between the Apple II+ and the PMF is accomplished through the interfaces described previouly. A simple flowchart of a sample control program is given in Figure 4. Program modifications are easily performed to accomodate specific applications.

Data is acquired by sequentially gathering the emission spectrum at different excitation wavelengths (Figure 1), as previously described. The TN-1710 multichannel analyzer contains 8 Kbytes (16 bits/byte) of RAM and therefore, can store up to 16 emission spectra at a time. After the data has been acquired by the TN-1710 it is then transferred to the Apple II+ and stored on mini-floppy disks as one-dimensional arrays. A list of the data acquisition and storage capabilities of the PMF are given in Table 2.

After the one-dimensional emission spectra have been stored, they can be recalled individually or accumulated into a two-dimensional matrix similar to that acquired by the VF. Figure 5 is such a two-dimensional spectrum acquired by the PMF. This capability provides the investigator with significant versatility. A complete two-dimensional spectrum can be rapidly generated when desired. However, for many applications only a few excitation wavelengths are needed, thus increasing the time resolution between spectra and

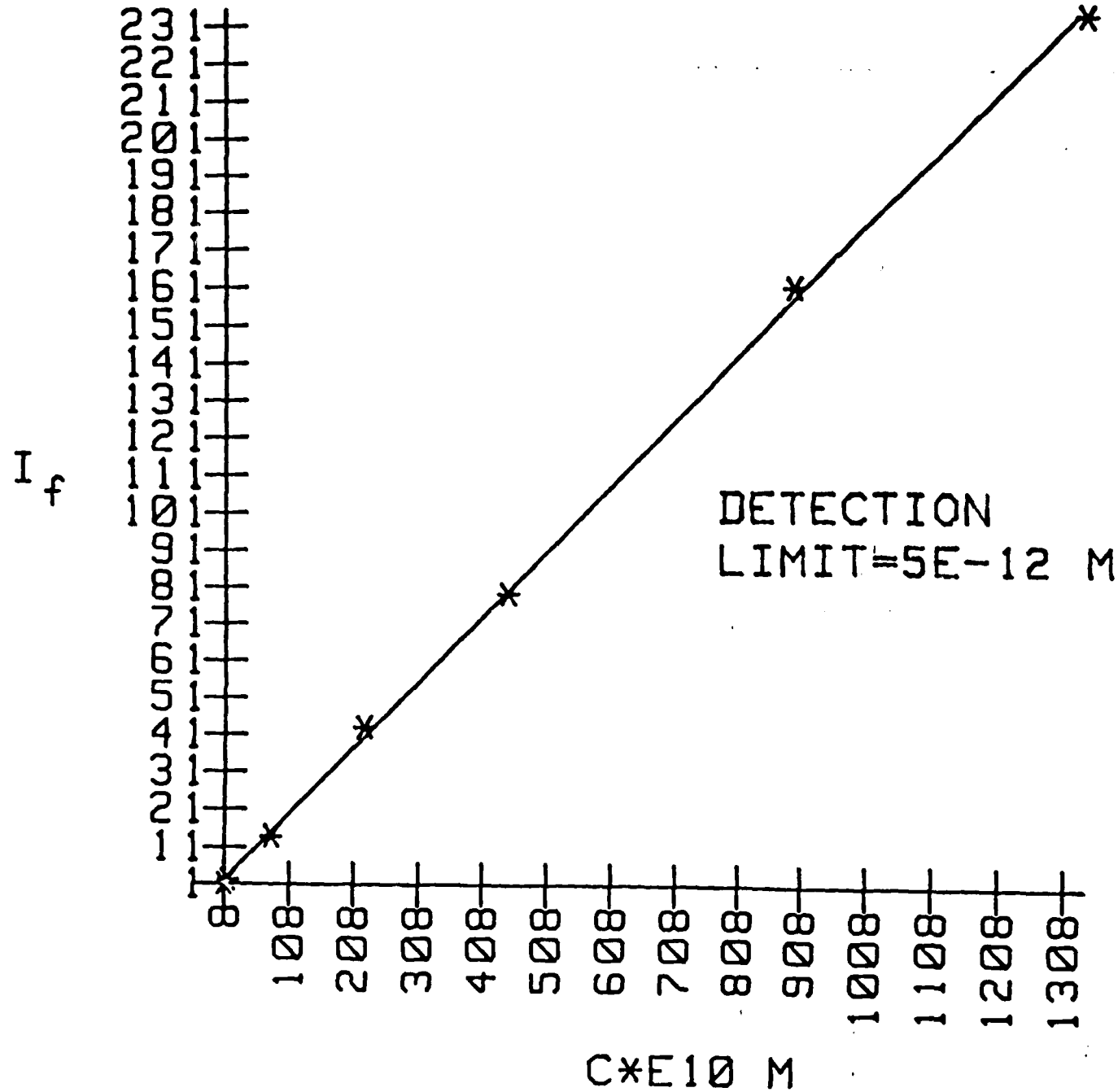
I_f
231
221
211
201
191
181
171
161
151
141
131
121
111
101
91
81
71
61
51
41
31
21
11
1
8
108
208
308
408
508
608
708
808
908
1008
1108
1208
1308
C*E10 M
DETECTION
LIMIT=5E-12 M

FIGURE 3

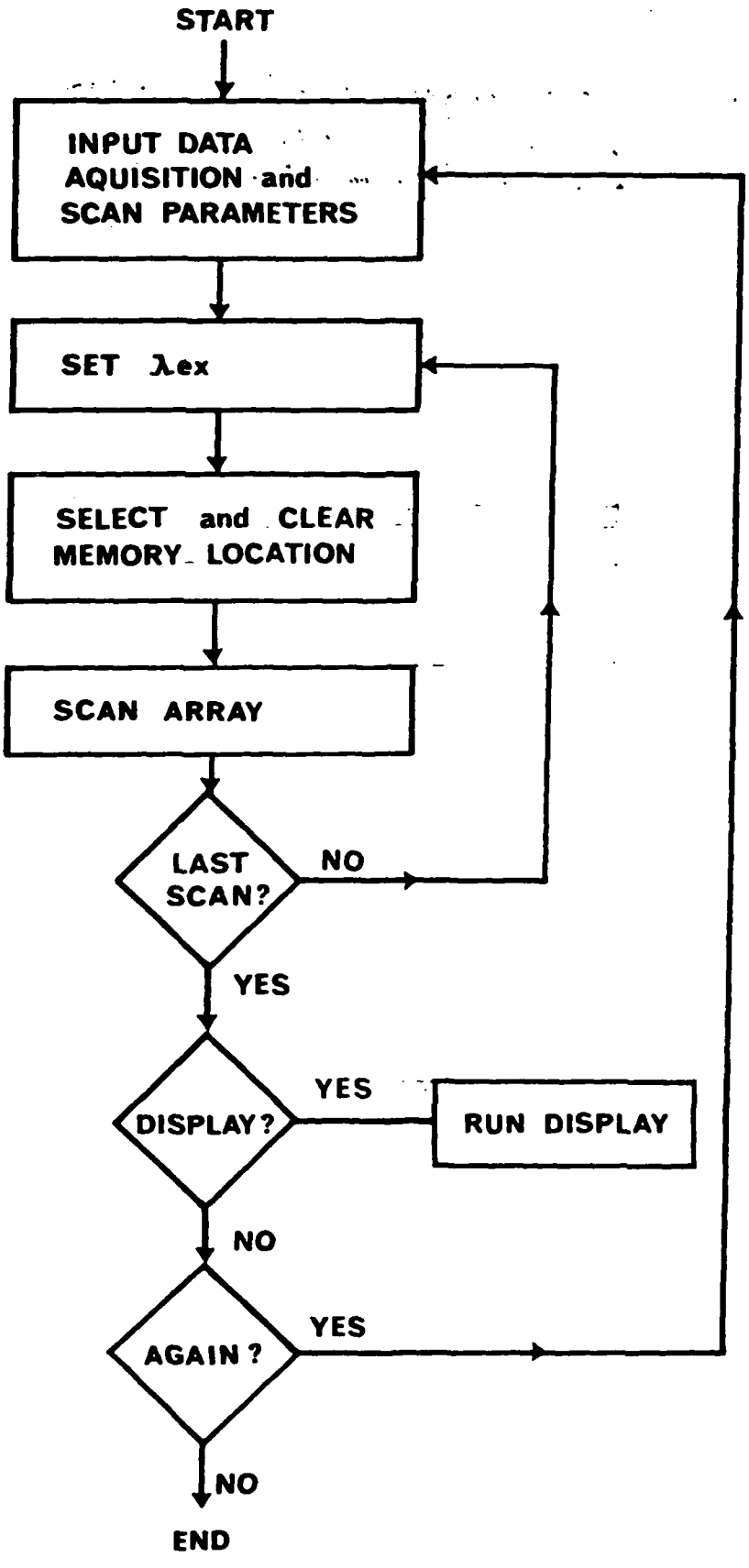
START
INPUT DATA AQUISITION and SCAN PARAMETERS
SET λex
SELECT and CLEAR MEMORY LOCATION
SCAN ARRAY
LAST SCAN?
NO
YES
DISPLAY?
YES
RUN DISPLAY
NO
AGAIN ?
YES
NO
END

FIGURE 4

Table 2

PMF data acquisition and storage capabilities

# of emission spectra	16
Data acquisition time (min.)	1-4
Data storage time (min.)	3
Total time (min.)	4-7
Storage capacity (spectra/disk)	98

reducing the data storage space required. FIG. 5

Preliminary Experiments

A total luminescence spectrum of standard chlorophyll a was acquired on the VF and displayed as an axonometric projection (Figure 6). There are five major excitation bands and one major emission band with a small longer wavelength maximum. The most intense excitation band, which occurs at 430nm, is called the Soret band. It is the Soret band which is usually monitored during in vivo chlorophyll a determinations and so the following data will focus on this excitation band. FIG 6

Characteristic spectra were acquired of the in vivo fluorescence of Chlamydomonas reinhardi which is a member of the Chlorophyta or "green algae" family. Spectra were also acquired of extracted chlorophyll a from real seawater samples. All of these spectra were obtained with the PMF for comparison with standard chlorophyll a spectra (Figures 7 and 8). Both of the extracted chlorophyll a spectra are similar to the standard spectra. However, the excitation band for the extracted sample is slightly broadened. This is likely due to the transfer of excitation energy from secondary pigments present in the phytoplankton to chlorophyll a which relaxes by fluorescence (Udenfriend, 1962). FIG 7-8

A significant difference between the excitation spectrum of the in vivo sample and the other two provides easy distinction. A 15-20nm red shift of the emission profile is also characteristic of in vivo fluorescence (Govindjee et al., 1973).

This preliminary data confirms previously reported results

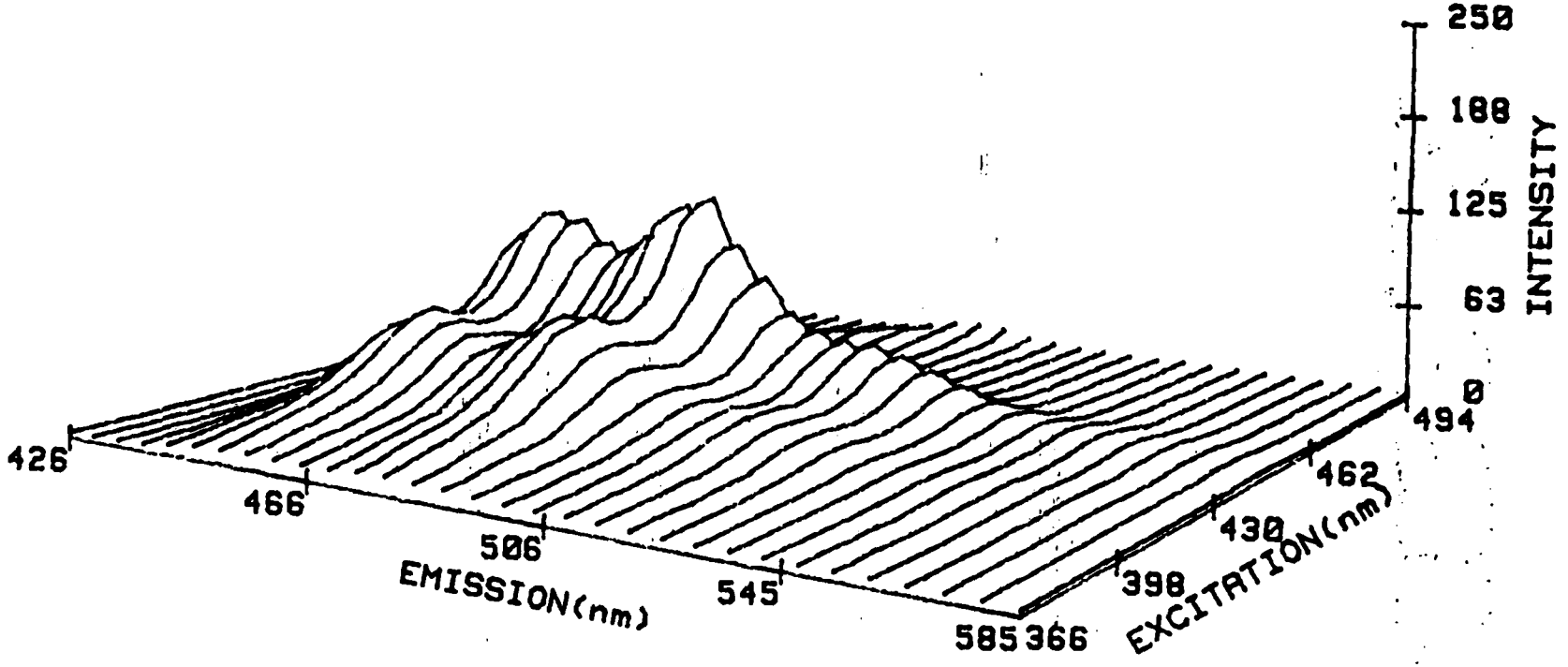
250
188
125
63
0
INTENSITY
426
466
506
545
585
EMISSION(nm)
366
398
430
462
494
EXCITATION(nm)

FIGURE 5

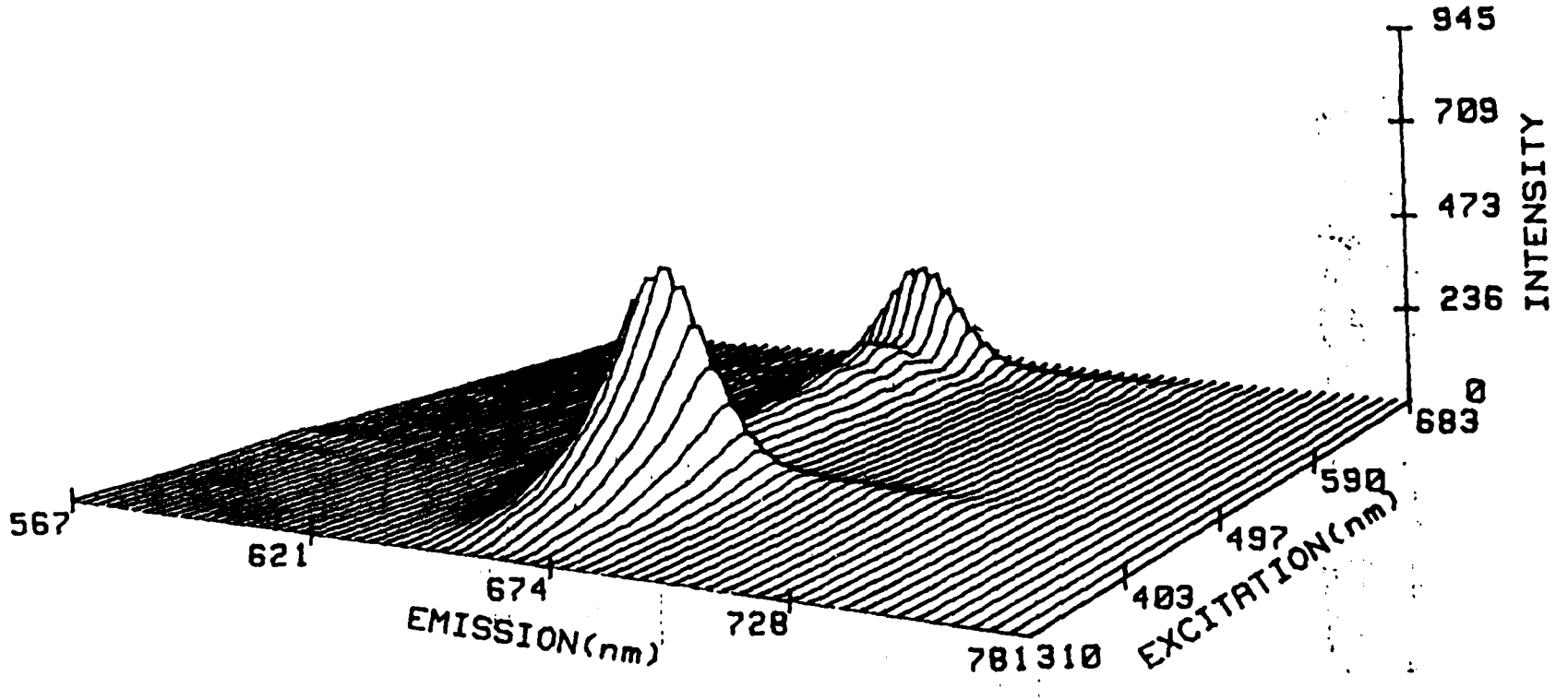
945
709
473
236
0
INTENSITY
683
590
497
403
EXCITATION(nm)
310
781
728
674
621
567
EMISSION(nm)

FIGURE 6

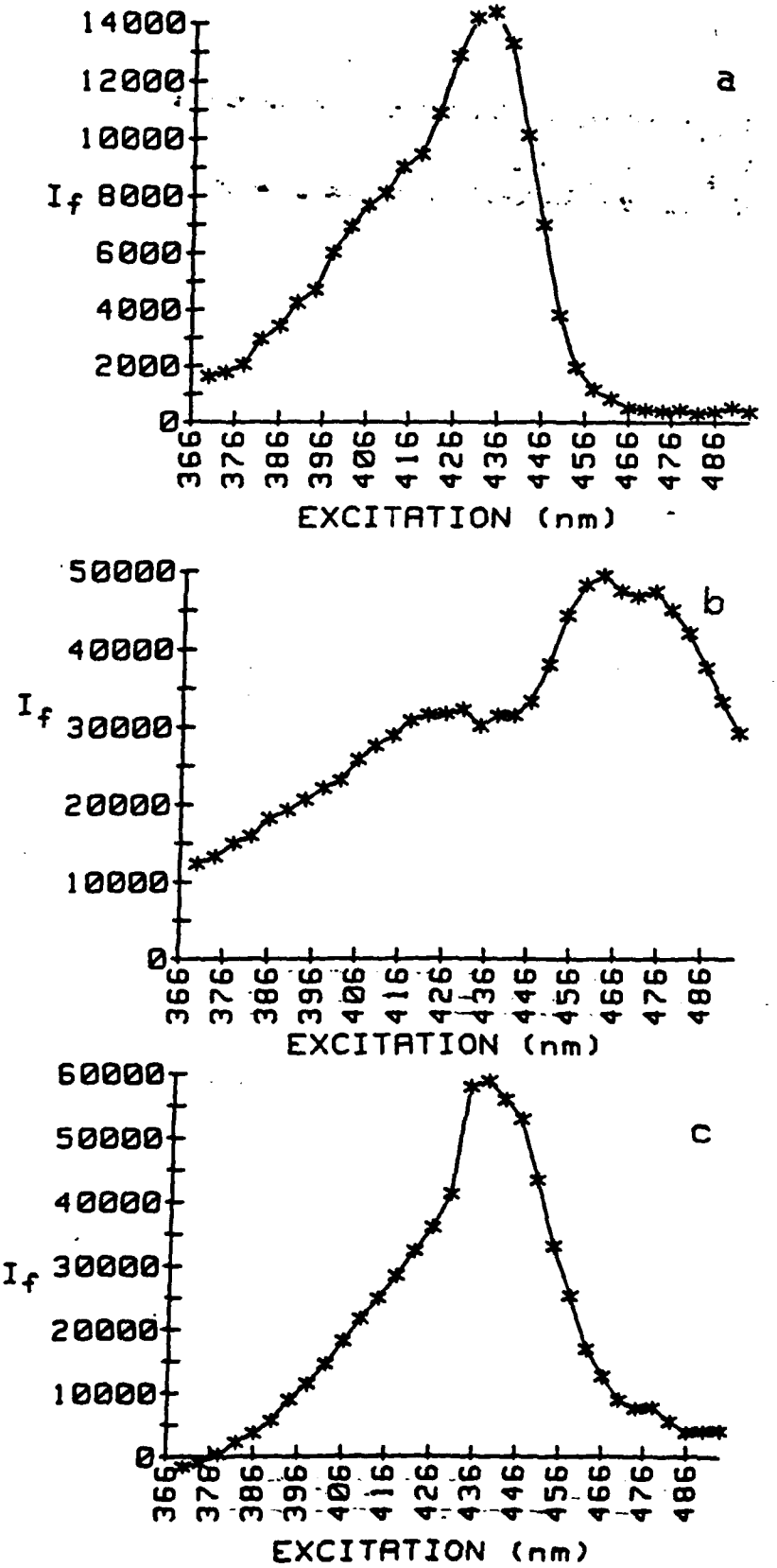
14000
12000
10000
I_f 8000
6000
4000
2000
0
366 376 386 396 406 416 426 436 446 456 466 476 486
EXCITATION (nm)
a
50000
40000
I_f 30000
20000
10000
0
366 376 386 396 406 416 426 436 446 456 466 476 486
EXCITATION (nm)
b
60000
50000
40000
I_f 30000
20000
10000
0
366 376 386 396 406 416 426 436 446 456 466 476 486
EXCITATION (nm)
c

FIGURE 7

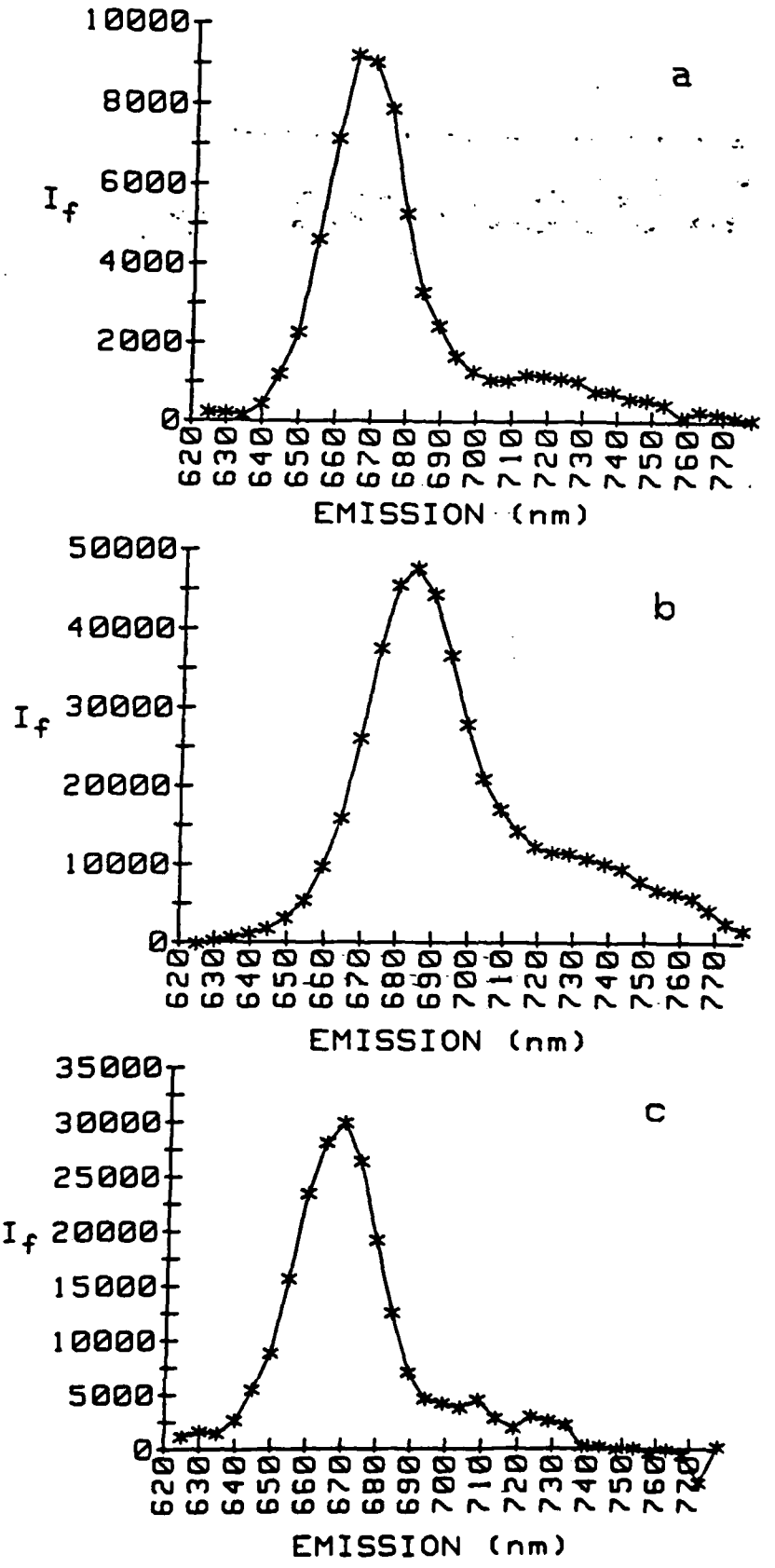
a
10000
8000
6000
4000
2000
0
I_f
620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770
EMISSION (nm)
b
50000
40000
30000
20000
10000
0
I_f
620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770
EMISSION (nm)
c
35000
30000
25000
20000
15000
10000
5000
0
I_f
620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770
EMISSION (nm)

FIGURE 8

(Yentsch et al., 1979) which indicate the excitation spectrum as a useful "fingerprint" of phytoplankton cultures. Further investigation is required to adequately determine the extent of spectral differentiation between phytoplankton cultures. The PMF provides the mechanism whereby the characterization of phytoplankton populations can be easily explored. It enables the continuous acquisition of "total luminescence" spectra useful not only for "fingerprinting" populations but also for detecting the spectral distribution of fluorescent species in relation to topography.

CONCLUSION

The characteristics of portability, sensitivity, rapid data acquisition, multidimensional detection, automation capabilities, and rugged construction have been provided in the design of a portable multichannel fluorometer (PMF). Conventional instruments are generally sensitive and possibly automated but lack the other attributes mentioned. It has been shown that multidimensional detection of fluorescence spectra is advantageous in multicomponent sample analysis. However, it is instrumentally limited by a lack of sensitivity and by restriction to laboratory investigations. The continuous determination of in vivo chlorophyll a as well as many other applications could benefit from a portable and sensitive fluorometer capable of rapidly acquiring multiwavelength spectra. Spectral information that is currently undetected could be useful in eliminating interferences and toward sample characterization. By incorporating an intensified photodiode array, a scanning circular variable filter wheel, and a controlling microcomputer such spectral information can now be

acquired. This fluorometer provides sensitive and rapid data acquisition and the capability of operating in a remote setting.

ACKNOWLEDGMENTS

The authors are grateful to Dr. D. R. Schink, S. T. Sweet, and P. J. Setser, all of the Department of Oceangraphy, Texas A&M University for their assistance in acquiring ship time and for useful discussions concerning chemical oceanography. This work was supported by the Office of Naval Research.

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FIGURE CAPTIONS

Figure 1. Method of excitation: Utilization of a circular variable filter wheel for sequential acquisition of emission spectra at different excitation wavelengths.

Figure 2. Diagram of the portable multichannel fluorometer (PMF).

Figure 3. Calibration curve and detention limit for chlorophyll a in acetone.

Figure 4. Sample BASIC program flowchart for data acquisition and control of PMF.

Figure 5. Two-dimensional fluorescence spectrum of perylene (10^{-8}M) acquired by the PMF and presented in an axonometric projection.

Figure 6. Axonometric projection of chlorophyll a (10^{-6}M) acquired by the VF.

Figure 7. Excitation spectra of a) standard chlorophyll a b) *in vivo* *Chlamydomonas reinhardi* c) extracted seawater sample.

Figure 8. Emission spectra of a) standard chlorophyll a b) *in vivo* *Chlamydomonas reinhardi* c) extracted seawater sample.

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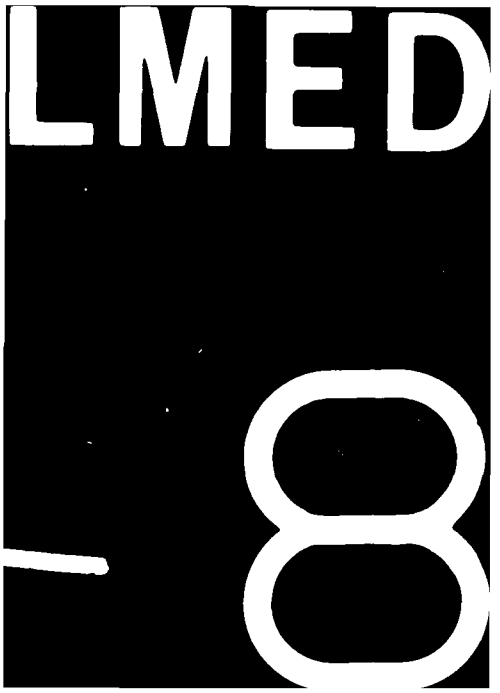
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